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Fluorescent protein recognition polymer thin films capable of selective signal transduction of target binding events prepared by molecular imprinting with a post-imprinting treatment

Hirobumi Sunayama, Tooru Ooya, Toshifumi Takeuchi*

Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

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ABSTRACT

The functional monomer bearing three functional groups for protein imprinting was designed, which has a structure consisting of a polymerizable methacryloyl group, a secondary amine group for fluorescent dye conjugation by a post-imprinting treatment, and a benzoic acid moiety capable of interacting with a target protein. Lysozyme-imprinted polymer thin films were prepared on the initiator-immobilized glass substrates by radical polymerization in the presence of lysozyme, the designed functional monomer, a co-monomer(s) and a crosslinker. After the removal of lysozyme, fluorescein isothiocyanate was introduced into the secondary amine group of the functional monomer residues in the imprinted thin film as a fluorescent reporter dye (post-imprinting treatment). Lysozyme was selectively bound to the thin film with a binding constant of ca. $10^6\ M^{-1}$. Since the reporter dye can be only introduced into the binding cavity, the fluorescent response can be detected only when the guest is bound to the cavity, namely only specific binding events can be transduced as fluorescence spectral change. Compared with the SPR measurement, selective binding to the imprinted cavity can be more precisely detected by the proposed method, enabling us to prepare a new class of protein recognizable materials with the ability of the specific signal transduction of protein binding events.

1. Introduction

Protein recognition is of great importance in the fields of life science, especially in the study of proteomics and diagnosis for cancers and other intractable diseases. Usually, biomolecules such as antibodies, enzymes, receptors, and various aptomers are used as protein recognition elements of these purposes. However, the preparation of such biomolecules are time- and cost-consuming, thus biomolecules mimics are desirable as substitutes, which have high selectivity and affinity for target molecules and can be inexpensively prepared with tailor-made fusion. Molecular imprinting is a template polymerization technique to prepare polymer adsorbents bearing pre-determined binding sites capable of recognizing target molecules. Molecularly imprinted polymers (MIPs) can be prepared by co-polymerization of functional monomers and crosslinkers in the presence of a template molecule (a target molecule to be recognized or its derivatives) (Komiyama et al., 2002; Haupt and Mosbach, 2000; Sellergren, 2001; Takeuchi and Haginaka, 1999; Turner et al., 2006; Vlatakis et al., 1993; Wei et al., 2006; Wulff, 1995; Zimmerman and Lemcoff, 2004). Recently targets of MIPs have been shifted from small molecules to proteins, and many studies have already been reported to prepare MIPs for proteins (Bossi et al., 2007; Hansen, 2007; Linares et al., 2009; Takeuchi and Hishiya, 2008; Zhao et al., 2009;).

Fluorescence has been frequently used as a highly sensitive technique for the detection and imaging of biologically active compounds, and naturally efforts have been made to introduce fluorescent binding sites to MIPs for the visualization of the binding events by measuring the fluorescence change (Tao et al., 2006; Kubo et al., 2005; Mitch et al., 2001). In most cases, fluorescent functional monomers were co-polymerized to construct polymer matrices, and the problem is that the polymers show high background fluorescence due to the intrinsic fluorescence of functional monomers that may be immobilized outside the specific binding cavity. To reduce such background, a minimum amount of fluorescent reporter molecule should be introduced into MIPs, which is located only inside the molecularly imprinted cavities. For example, Tao et al. (2006) has reported protein-imprinted xerogels, where an azido-fluorescent dye was conjugated near the binding sites by photoreaction.

We have developed a novel way to introduce a fluorescent reporter molecule to the binding sites in hydrogel thin films by using a newly designed functional monomer **1** and a post-imprinting treatment (McNiven et al., 1997; Mukawa et al., 2002;

* Corresponding author. Tel.: +81 78 803 6158; fax: +81 78 803 6158.
E-mail address: takeuchi@gold.kobe-u.ac.jp (T. Takeuchi).

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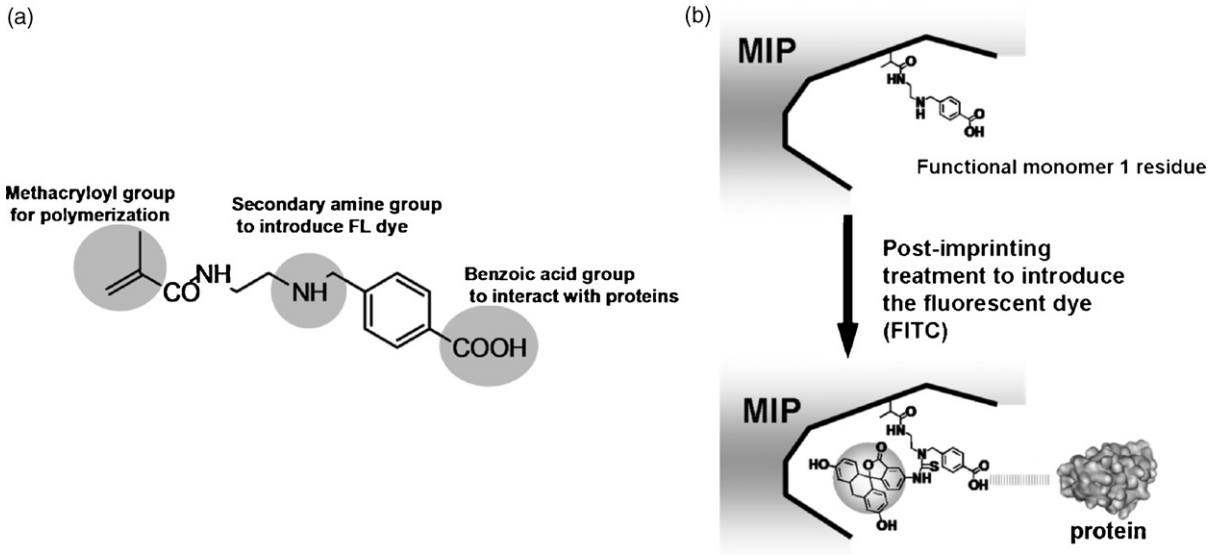

Fig. 1. (a) Concept of newly designed functional monomer **1**; (b) schematic drawing of the post-imprinting treatment to prepare fluorescent protein recognition cavity.

Murakami et al., 2008; Takeda et al., 2009; Takeuchi et al., 2006; Yane et al., 2006) with an amino-reactive fluorescent dye (Fig. 1). In this study, lysozyme (Mw: 14,300, pI: 11.2) was used as a model target molecule, and as a fluorescent reporter molecule, fluorescein was employed. Fluorescent MIP thin films were constructed on glass substrates and the signal transduction of protein binding events was evaluated by measuring the fluorescence spectral change. In addition, the binding behavior of the target and reference proteins was investigated by surface plasmon resonance (SPR) measurements for the MIP thin films prepared on the SPR sensor chips.

2. Experimental

2.1. Materials

Ethylenediamine, *N*,*N*′-diisopropylethylamine (DIEA), *4*-folmylbenzoic acid, triethylamine (TEA), citric acid, sodium hydrogen carbonate, diethylether, methanol, and dichloromethane were purchased from Nacalai Tesque (Kyoto, Japan). Methacrylic acid, acrylic acid, acrylamide, 2,2′-azobis(2-methylpropioneamidine)dihydrochloride (V-50), *N*,*N*′-methylenebisacrylamide (MBAA), and tris(hydroxymethyl)aminomethane were purchased from Wako Pure Chemical Industries (Osaka, Japan). Di-*tert*-butyldicarbonate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDC·HCl) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). *N*-Hydroxy succinimide (NHS), 11-mercapto undecanoic acid, lysozyme, cytochrome c, myoglobin, lactalbumin were purchased from Sigma–Aldrich (USA). 4 M HCl in dioxane and sodium triacetoxyborohydride were purchased from Tokyo Chemical Industry (Tokyo, Japan). Triethoxysilylpropyl maleamic acid was purchased from AZmax (Chiba, Japan). Dichloromethane was distilled prior to use.

2.2. Synthesis of the designed functional monomer ***1***

2.2.1. Preparation of (2-aminoethyl)carbamic acid tert-butyl ester (***a***)

Di-*tert*-butyl dicarbonate (1.1 g, 5 mmol) dissolved in dichloromethane (20 mL) was added dropwise to a solution of ethylenediamine (3.5 mL, 50 mmol) in dichloromethane (50 mL) over 1 h with vigorous stirring at 0 °C. Stirring was continued for a further 12 h at room temperature. The reaction mixture was washed with saturated NaCl aq and pure water. Then, the solvent was evaporated and dried *in vacuo* to obtain (**a**) as a colorless and clear liquid.

Yield: 70.0%

^{1}H NMR (300 MHz, $CDCl_3$): 4.85 (br, 1H, carbamide), 3.18 (t, 2H, $-CH_2-$, J = 6.0), 2.79 (t, 2H–CH_2-, J = 6.0), 1.45 (s, 9H, Boc), 1.29 (br, 2H, $-NH_2$).

2.2.2. Preparation of (2-methacryloylaminoethyl)carbamic acid tert-butyl ester (***b***)

Methacrylic acid (4.92 μL, 6 mmol) and EDC·HCl (1375 mg, 7.2 mmol), DIEA (1894 μL, 13 mmol) were dissolved in dichloromethane (20 mL). The reaction mixture was stirred under nitrogen atmosphere at 0 °C. Then (**a**) (480 mg, 3 mmol) dissolved in dichloromethane (30 mL) was added to the mixture and stirred for 4 h. Stirring was continued for a further 12 h at room temperature. The reaction mixture was washed with $NaHCO_3$ aq, Citric acid aq, and pure water. Then, the solvent was evaporated and dried *in vacuo* to obtain (**b**) as a white solid.

Yield: 51.2%

^{1}H NMR (300 MHz, $CDCl_3$): 6.69 (br, 1H, amide), 5.76 (s, 1H, vinyl), 5.33 (s, 1H, vinyl), 4.91 (br, 1H, carbamide), 3.43 (t, 2H, $-CH_2-$, J = 5.1), 3.34 (t, 2H, $-CH_2-$, J = 6.0), 1.96 (s, 3H, $-CH_3$), 1.44 (s, 9H, Boc).

2.2.3. Preparation of N-(2-aminoethyl)metacrylamide hydrochloride (***c***)

Compound (**b**) (460 mg, 2 mmol) was dissolved in dichloromethane and stirred at 0 °C. Then, 4.0 M HCl/dioxane (2 mL, 8 mmol) was added to the solution and stirred for 12 h. The hydroscopic salt was filtered and washed with diethyl ether to obtain (**c**) as a white solid.

Yield: 76%

^{1}H NMR (300 MHz, CD_3OD): 5.78 (s, 1H, vinyl), 5.45 (s, 1H, vinyl), 3.51 (t, 2H, $-CH_2-$, J = 6.0), 3.07 (t, 2H, $-CH_2-$, J = 6.0), 3.45 (t, 2H), 3.33 (t, 2H), 1.96 (s, 3H, $-CH_3$), 1.29 (s, 2H, $-NH_2$)

2.2.4. Preparation of 4-[2-(N-methacrylamido)ethylaminomethyl]benzoic acid (functional monomer ***1***)

Compound (**c**) (130 mg, 1 mmol) and TEA (280 μL, 2 mmol) were dissolved in methanol. Then, *4*-folmylbenzoic acid (150 mg,